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Communication to the Editor

Molecular Biology as Virtual Biology: Limitations of Molecular Biology in Pesticide Discovery*

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Abstract: Much discussion has focused upon the concept that a 'rational', molecular biology-based strategy could revolutionize pesticide discovery. The personal viewpoint presented here is that such a concept is fundamentally flawed because it is based on false assumptions about the extent to which biological systems are genetically determined. It is argued that, even at the very low level of biological complexity represented by the structures and functions of individual proteins, the predictive capacity of molecular biology remains too weak to form a reliable basis for industrial research strategy, even when coupled with almost unlimited computing power. Expectations that molecular genetics could somehow speed up the discovery process and replace other methods of enquiry may thus be over-optimistic and an awareness of its limitations is essential to avoid the wasteful allocation of research resources. This article aims to highlight some of the limitations of which those who are not actively engaged in molecular biology may be unaware and which some of those who are so engaged may choose to ignore. Comments and counter-arguments are actively invited.

Keywords: Rational design, drug discovery, molecular target sites, gene function, genetic determinism.

1 INTRODUCTION

'Genes have created us, body and mind'.[1]

'I don't want to miss out on learning how life works'.[2]

'It will transform our capacities to predict what we may become'.[3]

'It may totally reshape the nature of *Homo sapiens*'.[4]

'With gene targeting you can do anything you want'.[5]

Pesticide discovery is an increasingly complex and expensive problem. As the cost of screening ever more compounds for economically viable biological activity mounts alarmingly, discussion has focused on the possibility of replacing random screening by a rational strategy in which molecular genetics is frequently cited as a key component.

Ever since the publication of the structure of DNA by Watson and Crick, molecular genetics has acquired an aura of almost fundamentalist certainty and mystique. The grandiose phraseology of some of its practitioners and the uncritical, almost missionary tone of reports in the press and popular scientific literature could easily give the impression that molecular genetics is the final key which will inevitably open the way to the rapid solution of all biological problems. The idea that biology is 'all in the genes' and ultimately determined by DNA alone, permeates through public, and even some scientific thinking, as an unquestionable dogma. This appears to have fostered the belief that the biology of organisms can be predicted from the sequence of

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their DNA, if only we study it intensively enough. Problems, it seems, need only wait for the intense light of molecular genetics to be focused upon them for the illumination offered by other forms of biological research to appear feeble, if not superfluous. A practical consequence of this has been the massive diversion of the funds available for academic biological research away from traditional methods of enquiry and into a molecular biology-based approach with a concomitant loss of many scientists with other types of valuable expertise. It is against this DNA-dominated intellectual background that discussions of the possible applications of such techniques in pesticide discovery have taken place.

There can be no doubt that molecular biology has proved to be a powerful tool in medical and biological research. However, while the effects in agriculture may also be profound, particularly via the creation of transgenic crop plants, it is not enough to assume that molecular biology could also revolutionize pesticide discovery, and simply set the molecular biologist to work. Precisely how such techniques could be applied to the search for new, biologically active compounds for use in agriculture needs to be assessed very carefully. It is possible that the spectacular successes of molecular biology and the rapid progress from discovering the structure of DNA to the creation of transgenic organisms have led to highly unrealistic expectations of what can be predicted from gene sequences.

At the risk of stating the obvious to those well versed in the topic, this communication aims to highlight some fundamental limitations of molecular genetics which may not be immediately apparent to those who lack the opportunity to study the primary literature, and to argue against the idea that a molecular genetics-based, rational approach could replace or dramatically reduce the necessity for other methods of biological enquiry. If the tone at times appears to be excessively negative, it is because the aim is to promote discussion and comment, particularly from the many, very able prophets of molecular genetics who preach the opposite point of view.

2 DEFICIENCIES OF THE 'MOLECULAR GENETIC' APPROACH

2.1 The biochemical functions of proteins encoded by the majority of identified genes are unknown

The identification of a gene associated with a disease or other biological process is often reported as if it were the single, most important breakthrough required to solve the problem. A vital fact which is not widely reported and which may thus escape the attention of those not actively engaged in such research is that the biochemical and physiological functions of the proteins encoded by the genes so identified are almost always unknown.

In its most extreme, simplistic form, molecular biology regards the cell as a molecular hierarchy in which all other molecules are subordinate to the dictates of DNA. Indeed, sociobiology extends this idea to the level of organisms and whole ecosystems and contends that life-forms are little more than the mechanisms by which genomes attempt to maximize the number of copies of themselves. Against such a background, it is tempting to believe that molecular biology can somehow by-pass the need for other methods of biological enquiry, as in the quote at the beginning of this communication in which it was claimed that 'With gene targeting you can do anything you want'. However, in the same interview, the prominent molecular biologist concerned had to concede that '... in each case we've been very poor at predicting, based on our previous knowledge of a gene, what the actual function of that gene is in a living organism'.[5]

Plant protection products usually disrupt the physiology of the target organisms by interacting with proteins, not genes. To be of any practical value in the search for new, biologically active compounds, the precise biochemical and physiological functions of the proteins encoded by identified genes must be known or no test systems can be established. The essential point for practical purposes is that a DNA sequence which encodes a protein of unknown function is little more than a string of letters. Computer-aided comparisons with sequences in databases may provide a basis for speculations about the possible functions of proteins encoded by genes, but without considerable investments of time and resources in confirmation by other techniques, they remain no more than pure conjecture. There are 'sequence motifs' which may suggest that a protein could bind, for example, calcium or NADH or may be phylogenetically related to other proteins, but, as this communication will try to illustrate, this is nothing but an academic computer game of 'virtual biology' and a very long way from defining the function of a protein with a level of confidence that could be of practical value in plant-protection research. In spite of the confident atmosphere which emanates from much molecular biological research, the gulf of ignorance between knowledge of a DNA sequence and knowledge of the precise functions of the protein it encodes usually remains extremely wide.

2.2 The structures of proteins cannot be predicted from their gene sequences

Establishing the central pillar of molecular biology, that the functions and structures of proteins are ultimately determined by the base sequences of the genes that encode them, was undoubtedly a great scientific achievement. This very success, often referred to in quasi-religious terms as 'the central dogma', appears to have fostered the belief that absolute determinism

extends beyond DNA-RNA-Protein and, even more misleadingly, that 'is determined by' is synonymous with 'can be predicted from'.

There may be a limited number of ways in which polypeptide chains can fold into stable conformations. This may explain the observation that many of the proteins so far crystallized tend to fall into groups, the members of which have remarkably similar three-dimensional structures. However, extremely similar three-dimensional protein structures can be formed by polypeptides with no detectable similarity in their gene or amino acid sequences.[6]

The simple, deterministic link between amino-acid sequence and three-dimensional structure is further undermined by a group of recently discovered proteins known as chaperonins.[7] These can bind to nascent polypeptides as they emerge from ribosomes and prevent them from aggregating or forming 'inappropriate' intramolecular bonds. When complete, the non-folded polypeptide is released into the cavity of a larger chaperonin complex, within which the final folding of the protein is directed by providing a controlled environment in which it can occur. Thus, a rather complex, cooperative biochemical process is required to ensure that sufficient yields of polypeptides fold to their functional conformations, rather than to other, non-functional possibilities which are thermodynamically equally likely. These findings call into question the view that protein folding is always a spontaneous process entirely determined by and, given sufficient data and computer power, predictable from knowledge of the primary amino acid sequence alone.

Therefore, although, in general terms, the possible three-dimensional conformations that can be adopted by polypeptide chains in any given environment may be strictly limited by their amino-acid sequences, their functional conformations cannot be predicted from them.

2.3 The functions of proteins cannot be predicted from their structures

As already noted, proteins, with no detectable sequence similarity, often fall into classes which can have remarkably similar three-dimensional structures. However, even high levels of structural similarity do not indicate any degree of functional similarity. At first sight, at least to the non-expert, the TIM-barrel fold protein domain, with its eight β-strands surrounded by eight α-helices, appears to be a three-dimensional form whose complexity could confer some degree of functional specificity. However, up to the year 1991 this structure had been found in proteins with 19 different biochemical functions[8] and the total may now be even greater. The structural similarity of these proteins is so great that domains of approximately 150 alpha carbon atoms of any two members of the group can usually be three-dimensionally superimposed to within a root mean square value of 2.5 Angstroms, yet, not only do database searches fail to reveal any sequence similarity, but their functions are also very diverse, ranging from those of the original triose phosphate isomerase to flavocytochrome b_2. Therefore, the three-dimensional structures of proteins offer few clues as to their possible functions.

2.4 Proteins with no detectable sequence or structural similarity can have identical functions

When organisms diverge and speciate during evolution this is reflected at the nucleic acid level and the DNA sequences encoding specific proteins also diverge. This can be valuable in phylogenetic studies, where the degree of sequence similarity of proteins with the same function in different organisms may be an indicator of the closeness or otherwise of their evolutionary relationships. However, as pointed out by R. F. Doolittle,[6] there are several instances of enzymes with precisely the same biochemical functions having arisen on separate occasions during evolution. Such proteins bear neither sequence nor structural resemblance to each other, in spite of catalysing identical reactions. Consequently, the complete absence of any detectable sequence or structural similarity does not exclude total identity of function.

We are thus in a situation where almost identical three-dimensional protein structures are formed by totally different amino acid sequences, almost identical structures can have completely different biochemical functions, and identical functions can be carried out by proteins with totally different sequences and structures. Predictions of structure and function based on sequence are thus of scarcely more practical value than saying that, because my dog has four legs and a tail and barks, the next animal I meet with this combination of limbs is also likely to bark. Although this prediction may, by chance, turn out to be true, it is wise to remember that a mouse also has four legs and a tail, as does a Bengal tiger. Far from being keys to the prediction of complex biological phenomena, such as controlling populations of plant pathogens, gene sequences alone offer only the most tentative and speculative guide to the very low level of biological complexity represented by the structures and functions of individual proteins. Except in the rare cases where homology with a protein of known function, preferably from a related species, is overwhelmingly great, gene sequence data have virtually no predictive power in terms of function. On the contrary, for practical purposes they are intrinsically uninterpretable without a great deal of additional physiological and biochemical information. Genome sequencing projects, and research which yields only gene sequences associated with biological observations, are thus unlikely to be of any direct value in pesticide research.

This is well illustrated by the publication of the complete DNA sequence of yeast chromosome III.[9] The 315-kilobase sequence revealed putative genes which may code for 182 proteins with chain lengths of 100 amino acids or more. Of these, 145 did not correspond to any known genes, though 29 showed some degree of similarity to sequences in databases. The number of these 29 database sequences, or of the 37 known genes identified which have been assigned precise biochemical functions was not stated. Such findings may open up exciting horizons for molecular biologists and stimulate a great deal of speculative sequence comparison, but whether or not they form a basis on which one could confidently justify the investment required for a substantial industrial research project is debatable.

2.5 Molecular genetics alone cannot identify new biochemical targets safely

It has often been suggested that molecular genetics could help to identify essential enzymes that could be new biochemical target sites for pesticides. Techniques frequently cited as being capable of this are anti-sense in plants and gene disruption in fungi. In anti-sense technology, the specific mRNA encoding the protein under investigation is inactivated by inducing the production of a complementary 'anti-sense' strand of RNA with which it combines. The double-stranded RNA cannot be translated to protein, so the amount of the target protein present in the plant can be greatly reduced, if not totally eliminated. In gene disruption, single genes can be specifically inactivated by the insertion of a non-coding section of DNA into their sequence. When this is carried out in a diploid yeast strain there will be instances when only one of the two copies of the gene in a cell has been disrupted. If the yeast is then induced to sporulate, each cell produces four ascospores, two of which will contain disrupted copies of the target gene. The spores can be separated using a micromanipulator and cultured individually.

If gene disruption prevents yeast spores from forming colonies, or the presence of a specific anti-sense RNA impairs plant growth, it could be argued that the proteins so identified are potentially good targets for fungicides and herbicides respectively. This may be the case, but there are good reasons for treating the results of such experiments with great caution. For example, genes encoding proteins essential for competitive advantage in the field, or for the infection processes of plant pathogens, may be non-essential under laboratory conditions or perhaps even totally absent from such model laboratory organisms as *Saccharomyces cerevisiae* Meyer ex Hansen. A possible example of this is melanin biosynthesis, a target of several rice blast fungicides.[10] Melanin biosynthesis is essential only during appressoria formation; neither spore germination nor mycelial growth is inhibited by these fungicides which, nevertheless, provide excellent protection from the disease. It is, therefore, doubtful whether gene deletion experiments would have revealed melanin biosynthesis as a potential target site for fungicides. There are fewer objections to the use of anti-sense to identify possible targets for total herbicides, but even this is not without problems, not least of which is the selection of a likely target.

Both anti-sense and gene disruption require significant investments of time and resources to investigate a single potential target. Unfortunately, there are no objective grounds on which to base the selection of any particular enzyme as a probable pesticide target and the choice is quite wide. Extrapolating from the 182 putative genes encoded by the 315 kilobases of yeast chromosome III, the complete yeast genome of *c.*14 megabases probably contains genes for a little more than 8000 proteins. This may be the minimum required to maintain eukaryotic cell organization and more complex fungi and plants could have many more.

The biochemistry of even a 'simple', unicellular organism such as yeast is a highly complex network of enzyme systems with many known, and a multitude of unknown interactions and feedback loops within and between pathways. Attempting to predict the enzyme that, if the gene encoding it were to be deleted, would not only lead to prevention of the growth of yeast cultures, but whose inhibition by chemical means would also be likely to control a range of fungal populations in the field, demands, to say the least, a high level of optimism, if not blind faith! A comparable problem would be attempting to predict which streets in London, Paris and Berlin should be blocked in order to bring the traffic in each city to a halt: should it be the ring road or perhaps that leading to the airport? This would be a difficult enough task in itself, but introducing the additional handicap of being armed with only a very incomplete street map of Ulan Bator as a guide, the odds are considerably lengthened.

What does a sensible industrial research scientist do in such a situation? The obvious answer is 'nothing'. Throughout the world there are thousands of researchers working on yeast genetics and anti-sense who make their results freely available in the literature which can be scanned for reports of lethal gene deletions. Of course, the majority of these deletions are of genes encoding proteins of unknown function, which, as already discussed, is of little direct practical value, but genes encoding proteins of known function have also been deleted.

2.6 Azole fungicides might never have been discovered if molecular biology had been used to identify their target site

There is an instructive example in the literature of the deletion of a gene encoding a protein of known function which is also known to be an important biochemical

target of fungicides. This allows a revealing, retrospective 'thought' experiment to be performed.

The gene in question is *erg 11*, which encodes the enzyme sterol 14α-demethylase,[11] a known target of many very effective azole fungicides and antimycotics.[12] The results of disrupting the *erg 11* in *S. cerevisiae* were as follows. When the ascospores were separated following the gene disruption procedure, those containing disrupted copies of the gene were initially unable to grow aerobically on nutrient agar and growth could be induced only under anaerobic conditions in liquid medium supplemented with ergosterol. This appears to be the successful identification of a target. However, within only two days, spores containing disrupted copies of the gene began to grow aerobically on non-supplemented agar. Sterol analysis revealed that the cultures contained only 14α-methyl sterols, so there had been no reverse mutation. Further genetic analysis revealed that an additional mutation in the sterol pathway which allowed aerobic growth in the absence of demethylase activity had occurred spontaneously. Similar disruption experiments with *Candida albicans* (Robin) Berkhout showed that this organism did not require the additional mutation for aerobic growth with a deleted *erg 11* gene.[13]

In view of all the concerns about resistance and the capacity of these organisms to grow quite vigorously in spite of the complete absence of any 14α-demethylase activity, it is doubtful that the sterol 14α-demethylase would have been identified as a potential target site by these experiments. The azole fungicides might thus never have been discovered had gene deletion been the main basis for the selection of a target site. The gene encoding yeast sterol Δ^{8-7} isomerase, a known target of the other main group of sterol biosynthesis inhibitors, the morpholines, has also been deleted and shown to be non-essential for the growth of yeast cultures.[14] Target identification by molecular genetic methods alone, especially when based solely on yeasts for which efficient transformation systems have been developed, is thus far too unreliable to provide definitive results on which to base decisions about potential target sites in pathogenic fungi.

2.7 Molecular screening systems cannot replace biological tests

Another aspect of the rational design approach to pesticide discovery frequently proposed is the use of a battery of cellular, molecular and enzyme-based prescreening systems which can be automated to test many thousands of compounds quickly and relatively cheaply. Obviously, such tests can be useful to optimize, for example, enzyme inhibition when a biochemical mode of action has already been identified. However, a highly specific test can yield only highly specific results and could not be used as a primary screen for activity in a complex system with any acceptable degree of confidence.

Structure/activity studies at the enzyme inhibitor level should also be approached with caution when used as a guide to biological activity, because high activity against a single target site may also be associated with high specificity. This may be desirable in pharmaceutical products where side-effects should be minimized, but may be counter-productive in plant protection where most effective pesticides probably have multiple molecular targets, interactions with which contribute to their overall influence on the system. Morpholine fungicides, for example, are known to inhibit at least two enzymes of the sterol biosynthetic pathway, the sterol Δ^{14} reductase and the Δ^{8-7} isomerase.[15] Attempts to improve the performance of fenpropimorph by concentrating on optimizing inhibition of the reductase produced several excellent inhibitors of this enzyme,[16] but none was any better than fenpropimorph in the field and some were considerably worse. The most active reductase inhibitors were found to have virtually no activity as inhibitors of the isomerase, (A. Akers, unpublished). Optimizing inhibition of one enzyme may thus increase specificity to the point where activity against other, possibly unknown, but important sites of action is completely lost. High specificity could also increase the risk of resistance problems, as may have been the case with the benzimidazole fungicides.

Enzyme-based test systems have long been used in the pharmaceutical industry, where, as already noted, high specificity may often be desirable and, in any case there are few alternatives for screening large numbers of compounds. The plant protection industry is much more fortunate; the number of plants damaged or killed in the search for new products has no moral consequences and, relative to animal research, screening on plants costs little and allows new compounds to be tested immediately in systems with high levels of biological complexity which can reveal novel biological activity without any prior knowledge of the associated biochemical mechanisms. It would be folly to forsake the advantage of screening compounds at the highest economically viable level of biological complexity in order to speed up the testing procedure if this could increase the danger that new, potential lead structures would be missed.

2.8 Protein crystallography may have little value in pesticide discovery

In the idealized scenario of a rational design approach to pesticide discovery, the determination of the structures of target proteins by X-ray crystallography and the use of these structures to direct the synthesis of inhibitors with the best 'fit' is often seen as the ultimate

aim of rational design. However, it is becoming increasingly accepted that such an approach to drug design may be rather naïve and the rigid, lock and key model of protein–ligand binding may be becoming somewhat 'rusted'.[17] Enzymes are extremely dynamic structures which can undergo large conformational changes when binding their substrates and during catalysis. Flexible ligands also undergo radical structural changes on binding to proteins. Crystal structures probably represent the lowest energy conformations of proteins under the crystallization conditions, which may not be the in-vivo conformation to which the pesticide binds; the pesticide itself is also likely to induce conformational changes. Attempting to design pesticides on the basis of crystal structures may thus be more likely to lead to frustration than success.

Furthermore, crystal structures provide very precise images of single proteins from individual species, but pesticides controlling only one species are unlikely to be economically viable, with the possible exception of some of the major insect pests. Identifying the genes encoding proteins with the same catalytic function from, say, five or six target organisms, isolating, crystallizing and solving the structures of the proteins they encode, then designing an inhibitor with optimal activity against all of them is simply too large a task to contemplate, even if it were possible. Additionally, high intrinsic activity against a biochemical target is only one, perhaps not always the most important, of the desirable properties of an effective pesticide.

2.9 Biological systems are dynamic and interactive, not rigidly deterministic

In some ways the biology of plant protection chemicals may be even more complex than that of pharmaceuticals, owing to the greater number of organisms involved and uncontrollable variables such as the weather. Consequently, it is a very common experience that the field performance of compounds with excellent activity in glasshouse tests turns out to be extremely disappointing, owing to factors introduced by the increased biological and physical complexity of the field situation; several conferences have been devoted exclusively to this apparent discrepancy. What, then, are the chances of bridging an infinitely greater gulf of complexity and of predicting the consequences of inhibiting the activity of a single gene product in the immense complexity of the field situation, where changing populations of diverse organisms, each with thousands of pleiotropic genes, dynamically interact with each other and their constantly changing environment?

3 CONCLUSIONS

The belief that biology is 'all in the genes' appears, in part, to have been encouraged by the discovery of the elegant simplicity of some aspects of life and heredity at the molecular level. In some quarters this appears to have led to a shift away from thinking in terms of the genetic components of complex biological phenomena to the belief that they are all, at least predominantly, if not entirely, ultimately genetically determined.

The social and political implications of such beliefs have been the subject of long and often vitriolic debate since before the time of Darwin.[18] Fortunately, industrial research is less clouded by ideology. The question is simply a matter of whether or not using simple genetic determinism as the primary guiding principle of research is likely to lead to the more rapid and economical discovery of new products. In the opinion of this author, it is more likely to be extremely wasteful, especially when biological phenomena already discovered and explored by other means are claimed as a triumph for molecular biology as soon as sequences of DNA which appear to be linked to the processes are discovered.

Nature, even in its relatively tamed agricultural form, remains an infinitely subtle and adaptable system, the productivity of which farmers aim to optimize. Plant protection chemicals provide some tools to assist in this optimization, but, ultimately, it is an empirical process. Molecular genetics may contribute to our understanding of how the application of chemicals leads to biological consequences, but it cannot predict those consequences before the chemicals are applied. The probable responses of an agricultural system to the introduction of a synthetic chemical must, as ever, be determined by patient trials. They are no more predictable from the genomes of the organisms involved than the weather is from the physical properties of air and water or the economy of a country from the share prices on its stock market at any given moment. The hubris of failing to acknowledge the immense complexity and flexibility of biological systems appears to have led the more fervent apostles of molecular genetics to make, in the opinion of this author, highly exaggerated claims and promises which, as this communication has tried to illustrate, fall far short of being fulfilled, even at the lowest levels of biological complexity.

Surely, delusions of the omnipotence of the genome are not universal in the molecular genetics community and perhaps stem as much from journalistic sensationalism as from the real opinions of mainstream molecular biologists themselves? It is to these scientists, who should be more aware than any layman of the fundamental limitations of their techniques, that one can pose the question of how, precisely, can molecular genetics contribute to the vital task of pesticide discovery?

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